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Characterization of *Campylobacter jejuni* strains from different hosts and modelling the survival of *C. jejuni* in chicken meat and in water

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ACADEMIC DISSERTATION

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Contents

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ABSTRACT

Campylobacter Spp are recognized as a major cause of bacterial food-borne gastroenteritis worldwide, with *Campylobacter jejuni* and *Campylobacter coli* being the most common species isolated in human infections (WHO, 2011). The number of registered cases of human campylobacteriosis in Finland has ranged from 3,796 cases in 2001 to 4,231 cases in 2011. The reported incidence in Finland in the last 10 years is higher than the European Union average.

In order to compare human, chicken and cattle *C. jejuni* isolates, the presence or absence of four nonubiquitous genes were determined so that they could be associated with the source of the isolate. First, we tested the presence of *dmsA*, which encodes a subunit of the putative tripartite anaerobic dimethyl sulfoxide oxidoreductase (DMSO/trimethylamine *N*-oxide reductase). Second, we detected *cj1585c*, which encodes another oxidoreductase. Third, the serine protease gene *cjj81176-1367/1371* was isolated. Fourth, γ-glutamyl-transpeptidase gene *ggt* was detected. We ascertained that *ggt* and *dmsA* are present more frequently in isolates obtained from humans and chickens, whereas *cjj81176-1367/1371* and *cj1585c* are the most common in bovine isolates.

Campylobacter jejuni is able to survive in different environments and in a wide range of temperatures. The study of *C. jejuni* inactivation in minced chicken meat and dug well water ascertain that the Weibull model could be applied optimally to the data to build a reliable prediction model for the survival of this microorganism as a function of temperature. The longest survival time found for *C. jejuni* in minced meat chicken was at the storage temperature of -20°C, and that of dug well water was at 4°C.

We analyzed the effect of different seasoning as dry marinade combinations on accelerating the reduction of *C. jejuni* counts on chicken drumsticks and observed a decrease of more than 1 log CFU/g. In addition, our results showed that using some fractions of potato protein in combination with food additives and sodium lactate obtained inactivation levels in excess than 1.66 log CFU/g. The most important *C. jejuni* counts reductions were always obtained within the first hours after the application of the seasoning combinations onto the chicken meat.

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications referred to in the text by Roman numerals I to IV:

I. González, M., M. Hakkinen M, H. Rautelin H, and Hänninen, M.-L. 2009. Bovine *Campylobacter jejuni* strains differ from human and chicken strains in an analysis of certain molecular genetic markers. Applied Enviromental Microbiology 75:1208-1210.

II. González, M., Skandamis P.N, and Hänninen, M.-L. 2009. A modified Weibull model for describing the survival of *Campylobacter jejuni* in minced chicken meat. International Journal of Food Microbiology 136: 52-58.

III. González, M., and Hänninen, M.-L. 2011. Reduction of *Campylobacter jejuni* counts on chicken meat treated with different seasonings. Food Control 22:1785-1789.

IV. González, M., and Hänninen, M.-L. 2012. Effect of antimicrobial resistance on survival of *Campylobacter jejuni* in well water. Application of the Weibull model for survival. Journal of Applied Microbiology 113 (2): 284-293.

ABBREVIATIONS

AMP	ampicillin
ATCC	American Type Culture Collection
APZ	acceptable prediction zone
bp	base pair
CBA	columbia blood agar
CIP	ciprofloxacin
cju34	gene encoding dimethyl sulfoxide oxidoreductase
cj1585c	gene encoding a putative oxidoreductase
cjj81176-13712	gene encoding a putative serine protease
CO_2	carbon dioxide
CFU	colony forming unit
D_{value}	decimal reduction time
EFSA	European Food Safety Authority
ERY	erythromycin
GBS	Guillain-Barre syndrome
ggt	γ-glutamyl transpeptidase gene
HUS	hemolytic uremic syndrome
K_{max}	maximum death rate
N_2	nitrogen
NaCl	sodium chloride
MAP	modified atmosphere packaging
mCCDA	modified charcoal cefoperazone deoxycholate agar
MIC	minimum inhibitory concentration
MLST	multilocus sequence typing
NAD	nicotinamide adenine dinucleotide
NADP	nicotinamide adenine dinucleotide phosphate
PCR	polymerase chain reaction
PFGE	pulsed field gel electrophoresis
R^2_{adj}	adjusted coefficient of determination
RAPD	random amplified polymorphic DNA
RMSE	root mean square error
ST	strain type
TET	tetracycline
VBNC	viable but non-cultivable
WHO	World Health Organization

1. INTRODUCTION

Human campylobacteriosis is an important enteric infectious disease that affects both industrialized and the less developed countries throughout the world (FAO, 2009). In many countries campylobacteriosis is a notifiable disease. The economic loss due to *Campylobacter jejuni* infection worldwide is likely to be well in excess of US $2 billion per year (CDC, 2009). *C. jejuni* accounts for more than 90% of the campylobacteriosis cases and *C. coli* is usually associated with only a minority of the illnesses (Olson *et al*., 2008). In addition to the human and economic costs of the acute infection are the chronic sequelae associated with campylobacteriosis (Altekruse *et al*., 1999).

C. jejuni is zoonotic, and therefore there are many animal species that serve as reservoirs for the human disease. The principal reservoirs for *C. jejuni* are the alimentary tracts of wild birds, and farm livestock such as chicken, turkey, cows, pigs, sheep, and goats, a variety of wild mammals, rodents and shellfish (Miller & Mandrell, 2005). However, animals rarely succumb to disease caused by this organism. Most human infections occur as single cases or small family outbreaks, and epidemics are uncommon (FAO, 2009).

Specific risk factors associated with poultry have included eating raw or undercooked chicken meat and handling raw chicken meat during food preparation (Hakkinen *et al*., 2009). Outbreaks of *Campylobacter* in developed countries are mainly caused by poultry, contaminated drinking water, and unpasteurized milk (Olson *et al*., 2008). In the European Union, 333 foodborne outbreaks were attributed to *Campylobacter* spp. in 2009, a figure which represents 6% of all reported foodborne outbreaks. These infections mainly came from contaminated drinking water or unpasteurized milk (EFSA, 2011). Underreporting of campylobacter infections is common in most countries and incidence rates only reflect the number of laboratory-confirmed cases. As a result, the true rate of infection is higher than the number of reported cases, and is estimated to range from 7.6 to 100 times higher (Wheeler *et al.,* 1999; Mead *et al.,* 1999; Samuel *et al*., 2004).

This work concentrates on three goals. First, to investigate the host association of *C. jejuni* isolates in cattle, chickens and humans by using four nonubiquitous genetic markers. The second aim was to study and model the survival of *C. jejuni* in different matrices such as minced chicken meat and dug well water as a function of the temperature on Colony Forming Unit (CFU) decline. Third, the objective was to find new marination compounds and combinations, which were efficient in the reduction of *C. jejuni* on chicken meat.

2. REVIEW OF LITERATURE

2.1 Historical background

The genus name *Campylobacter* was derived from the Greek words "Campylo" (curved) and "bacter" (rod). The early history of this class of gram-negative bacteria started in 1886, when Dr. Theodor Escherich in his bacteriological research at the St Anna Childrens Clinic (Vienna) observed time nonculturable spiral-shaped bacteria in the colons of diarrheic dead infants for the first time. These infections were named cholera infantum or summer complaint (Kirst, 1985). In (1913), John McFadyean and Stewerd Stockman obtained a pure culture of a *Vibrio*-like organism from aborted ovine fetuses, which we now refer to as *Campylobacter fetus*. In (1919), Smith and Taylor isolated the same kind of organisms, which also caused vibrionic abortion in cattle (Smith & Taylor, 1919). The clinical relevance of *Campylobacter* spp. in humans was realized when in 1938, Levy observed that a spiral organism highly similar to "*Vibrio jejuni*" and which is now known as *C. jejuni* was responsible for an outbreak of gastroenteritis in two adjacent institutions in Illinois, USA (Levy, 1946). A few years later, King reported that "*Vibrio fetus*" was involved in bloodstream infections in humans (King, 1957). In 1963, after it was understood that the organisms differed from *Vibrio* spp., by their low DNA base composition, their microaerophilic growth requirements and their nonfermentative metabolism, *Vibrio fetus* and *Vibrio bubulus* species were reassigned into the new genus of *Campylobacter* as *Campylobacter fetus* and *Campylobacter bubulus*, respectively, thus the novel genus *Campylobacter* was established (Sebald & Veron 1963; Skirrow, 1977; Butzler, 2004).

The role of *Campylobacter* as an enteric pathogen was not discovered until 1970s, which was mainly due to the difficulty of isolating and cultivating these bacteria from fecal samples. In 1973, Véron and Chatelain published a more comprehensive study on the taxonomy of the microaerophilic *Vibrio*-like organisms, in which they investigated four distinct species in the genus *Campylobacter*: *C. fetus* (type species), *C. coli* isolated from feces of pigs with diarrhea (Doyle, 1948), *C. jejuni* isolated from feces of cattle with diarrhea (Jones *et al*., 1931), blood cultures of humans with

gastroenteritis (King, 1957) and aborted sheep fetuses (Bryans *et al*., 1960) moreover, two subspecies of *C. sputorum*: one of which, the subspecies *sputorum*, was isolated from the sputum of a patient with bronchitis (Prévot, 1940); whereas the other subspecies *bubulus*, was isolated from bovine vagina and semen (Florent, 1959).

The development of new adequate isolation procedures led to a renewed interest in *Campylobacter* during the 1970s. These procedures were i.e. filtration technique (Steele *et al*., 1984) and selective media (Skirrow, 1977), which led to the isolation of a plethora *Campylobacter*-like organisms from a variety of human, animal and environmental sources. Furthermore a new species was described (Lawson *et al*., 1981; McClung *et al*., 1983; Neill *et al*., 1985; Fox *et al*., 1989). Finally, the genera *Campylobacter* and *Arcobacter* were used to accommodate the new bacterial family *Campylobacteraceae* (Vandamme *et al*, 1991) that shared similar phenotypic and genotypic features.

In 1978 the first case of campylobacteriosis was reported in Finland (Kosunen, 1978).

2.2 Genus *Campylobacter*

To date, the genus *Campylobacter* comprises 25 validated species (Table 1); many of these are human or animal pathogens (Debruyne *et al*., 2008). The species type of the *Campylobacter* genus is *Campylobacter fetus*, which was formerly known as *Vibrio fetus* (Smith & Taylor, 1919). Within the genus *Campylobacter*, the group of thermophilic species, currently includes *C. jejuni*, *C. coli*, *C. helveticus*, *C. upsaliensis*, *C. lari*, *C. insulaenigreae*, *C. avium*, *C. peloridis, C. volucris* and *C. subantarcticus*, all of which form a distinct 16S rRNA phylogenetic subcluster. *C. fetus* and *C. hyointestinalis* are also close relatives, whereas the remaining species form a loose assemblage of predominantly hydrogen-requiring organisms.

Table 1. Reservoirs for *Campylobacter* spp. (Man, 2011)

Campylobacter spp.	Reservoir	1st description Reference	Pathogenecity in humans (+)	Pathogenecity in animals (+)
C. avium	Poultry	(Rossi *et al*., 2009)	?	?
C. canadensis	Whooping crane	(Inglis *et al*., 2007)	?	?
C. coli	Bird, Cattle, chicken, goat, human, swine, seagull, sheep	(Doyle, 1948)	+	+
C. concisus	Cat, dogs, human	(Tanner *et al*., 1981)	+	?
C. cuniculorum	Rabbit	(Zanoni *et al*., 2009)	?	?
C. curvus	Dog, human	(Tanner *et al*., 1984)	+	?
C. fetus				
ssp. fetus	Cattle, horse, kangaroo, sheep	(Smith & Taylor, 1919)	+	+
ssp. venerealis	Cattle	(Florent, 1959)	+	+
C. gracilis	Dog, human	(Tanner *et al*., 1981)	+	?
C. helveticus	Cat, dog	(Stanley *et al*., 1992)	+	+
C. hominis	human	(Lawson *et al*., 2001)	+	?
C. hyointestinalis				
subsp. hyointestinalis	Cattle, dog, human, swine, hamster, reindeer, sheep	(Gebhart *et al*., 1985)	+	+
subsp. lawsonii	Swine	(On *et al*., 1995)	?	?
C. insulaenigrae	human, elephant-seal, porpoise carcass, sea lion, wild common seal	(Foster *et al*., 2004)	+	?
C. jejuni				
ssp. doyley	Human	(Steel & Owen, 1988)	+	?
ssp. jejuni	Birds, cattle, chicken, dog, insects, swine, rabbit, water	(Jones *et al*., 1931)	+	+
C. lanienae	Cattle, human, pig, sheep	(Logan *et al*., 2000)	?	?
C. lari				
ssp. concheus	Human, mullusk	(Debruyne *et al*., 2009)	?	?
ssp. lari	Bird, cattle, cat, chicken, dog, horse, mollusc, monkey water	(Benjamin *et al*., 2003)	+	+
C. mucosalis	Dog, Pig	(Lawson & Rowland, 1974)	?	+
C. peloridis	Human, shellfish	(Debruyne *et al*., 2009)	?	?
C. rectus	Human	(Tanner *et al*., 1981)	+	?
C. showae	Dog, human	(Etoh *et al*., 1993)	+	?
C. sputorum				
ssp. bubulus	Cattle, human, swine, sheep	(Debruyne *et al*., 2009)	?	?
ssp. sputorum	Cattle, sheep	(Prévot, 1940)	+	?
C. subantarcticus	black-browed albatross, gentoo penguin, gray-headed albatross	(Debruyne *et al*., 2009)	?	?
C. troglodytis	Chimpanzee	(Kaur *et al*., 2011)	?	?
C. upsaliensis	Cat, dog, human	(Sandsted & Ursing, 1991)	+	+
C. ureolyticus	Horse, human	(Jackson & Goodman, 1978)	+	?
C. volucris	Black-headed gull	(Debruyne *et al*., 2009)	?	?

Members of the genus *Campylobacter* are slender, spiral, curved, gram negative rods and do not form spores. Cells in old cultures (more than 48 h of incubation) or after long air exposure may be present as coccoid forms, which are considered to be

degenerative forms. The size of the cells varies between 0.2 to 0.8 μm wide and 0.5 to 5 μm long. They are typically motile, with a characteristic corkscrew-like motion that is achieved by means of a single polar unsheathed flagellum at one or both ends of the cell. However, the cells of some species of the genus are nonmotile (*Campylobacter gracilis*) or have multiple flagella (*Campylobacter showae*). *Campylobacter* species grow under a microaerobic atmosphere and have a respiratory and chemoorganotrophic type of metabolism. Energy is obtained from amino acids or tricarboxylic acid cycle intermediates, but not directly from carbohydrates. Carbohydrates are neither fermented nor oxidized. Central physical limits for growth of *C. jejuni* are shown in Table 2.

Table 2. Physical limits for growth of *C. jejuni* (Roberts *et al*., 1996; AFSSA, 2006).

Parameter	Range	Growth Optimum	Growth inhibition
T (°C)	32-45	40-42	< 30 - > 45
pH	4.9-9.0	6.5-7.5	< 4.9 - > 9.0
O_2(%)	-	3-5	>15
CO_2(%)	-	10	-
Water activity (a_w)	-	0.997	<0.987
NaCl(%)	-	0.5	>2

C. jejuni is sensitive to various environmental stresses, including high-oxygen conditions, UV light, high salt concentrations, heat and low pH (Park, 2002). *C. jejuni* does not possess genes involved in cold-shock protein responses, and the inability to grow at low temperatures can be due to the absence of these protective proteins (Park, 2005). *C. jejuni* is susceptible to low pH and are killed readily at pH 2.3 (Blaser *et al*., 1980).

The size of genome of *C. jejuni* is approximately 1.6 Mbp, the GC content of *C. jejuni* is about 30% and the percentage coding of the bacterial DNA is about 93%. The sequence of *C. jejuni* genome is variable. The distribution of eight variable sequence regions has demonstrated that they are important components of the capability to adapt to variable external conditions. According to the Multilocus Sequence Typing (MLST), the correlation between clonal complex and the distribution of the genes is strong (Hepworth *et al*., 2007). The MLST data collected to date show that *C. jejuni* is

highly diverse with a total of 5746 distinct STs from some 16394 isolates deposited in the pubMLST database (http://pubmlst.org/campylobacter/28.2.2012).

2.3 Subtyping of *C. jejuni*

Epidemiological studies of *C. jejuni* describe a wide range of phenotypic and genotypic typing methods that have been developed in order to understand the special characteristics of these pathogens and to be able to trace their source. Subtyping beyond the species is important in collecting information on the relative weight of different sources in human campylobacteriosis (Dingle *et al*., 2001; Hald *et al*., 2004; Strachan *et al*., 2009; de Haan *et al*., 2010).

2.3.1 Phenotypic methods for subtyping *C. jejuni*

Phenotypic methods for subtyping *C. jejuni* include serotyping with heat-stable (Penner & Hennessey, 1980) or heat-labile antigens (Lior *et al*., 1982), phage typing (Salama *et al*., 1990), and biotyping (Bolton *et al*., 1984). The phenotypic methods, in particular the two serotyping systems had been used worldwide in laboratories, especially for the surveillance of a large number of isolates and outbreaks, but they have since been widely replaced by certain genotyping methods.

2.3.2 Genotypic methods for subtyping *C. jejuni*

Genotypic methods for subtyping *C. jejuni* are usually selected in order to improve the discrimination between the isolates for epidemiology surveillance purposes. Some of the most commonly used genotypic methods for *Campylobacter* are pulsed-field gel electrophoresis (PFGE), ribotyping, flagellin gene typing, random amplified polymorphic DNA typing (RAPD), and (MLST) (Wassenaar & Newel, 2000; Dingle *et al*., 2001; Wareing *et al*., 2003).

PFGE and certain other subtyping methods are used to trace the source of campylobacter to understand the epidemiology of campylobacter infection outbreaks and impact of the different potential sources. PFGE is more discriminatory than MLST and therefore is considered more suitable for short-term epidemiological

studies and for the determination of the source of investigation in outbreak situations (Maiden *et al*., 1998; Mickan *et al*., 2007; McTavish *et al*., 2009). Unlike PFGE, MLST is used successfully in long-term epidemiological studies and in deciphering the population structure of *Campylobacter* on a global scale (Dingle *et al*., 2005; McTavish *et al*., 2008; de Haan *et al*., 2010). Moreover MLST is used for studies of population genetics and chooses partial sequences of seven selected housekeeping genes.

2.4 *Campylobacter jejuni* reservoirs

Campylobacter jejuni is susceptible to a variety of environmental conditions that make it unlikely to survive for long periods of time outside the host. The bacterium does not grow at temperatures below 30°C (Table 2), which indicates that typically no growth is usually possible outside the host. The principal reservoirs of *C. jejuni* include poultry (chickens, turkeys, ducks, and geese), which carry *Campylobacter* as part of their gut microbiota (Beery *et al*, 1988). They have also been isolated from sea water, lake water, streams, rivers and estuaries that had been subjected to fecal contamination. *C. jejuni* populations have been shown to differ among host species and environmental niches. However, the relative contributions by the various possible sources of infection in humans using source attribution models are unclear (Mc Carthy *et al*., 2007).

2.5 Survival of *C. jejuni*

Different studies have shown that *C. jejuni* is a fastidious organism that requires advanced cultivation conditions *in vitro* and is able to survive for prolonged times in different habitats outside of the intestine. Temperature is the key factor for prolonged survival. Usually the most prolonged survival for *C. jejuni* occurs at refrigerator temperatures: not at room temperature (Bhaduri & Cottrell, 2004)

Water is one of the main transmission routes of campylobacteriosis (Koenraad *et al*., 1997). *Campylobacter* is a waterborne pathogen, which can survive for extended periods in natural water bodies after deposition by animal hosts, in the form of viable

but non-culturable (VBNC) cells (Rollins & Colwell, 1986). Guillou *et al*. (2008) could detect viable cells of *C. jejuni* in mineral water by CBA plate counts that had been stored at 4°C in the dark for 48 days. Cook & Bolster (2007) observed that the culturability of *C. jejuni* incubated in the dark at 4°C in filter-sterilized groundwater microcosm decreased below detection limits (20 cells/ml) within 85 days, regardless of the source or of the nutrient composition of the water.

Mihaljevic *et al*. (2007) observed that *C. jejuni* in ground chicken meat that had been refrigerated at 4°C could be detected after one week and also when chicken meat was stored at -20°C for two weeks. In other studies in which minced chicken meat was naturally contaminated and stored at refrigeration temperatures (3-4°C) for seven days, it was observed that the reduction of *C. jejuni* was 0.3 CFU/g (Georgsson *et al*., 2006; Sampers *et al*., 2010).

2.6 Sources of human *C. jejuni*

Campylobacter spp. are frequently isolated from foods of animal origin. The bacteria can readily contaminate various foodstuffs, including poultry chicken meat, raw milk and dairy products, and less frequently fish and fishery products, mussels and fresh vegetables (Kärenlampi & Hänninen, 2004). Human food can be contaminated at any point in the production-retail chain (Neimann *et al*., 2003). Among sporadic human cases, contact with live poultry, consumption of poultry meat, raw milk and untreated drinking water have been identified as important sources of *C. jejuni* infection (Schönberg-Norio *et al*., 2004). Even one drop of juice from raw chicken meat can be sufficient to infect a person (Birk *et al*., 2004). Poultry meat products appear to be one of the major sources of campylobacteriosis. One common way by which the bacterium is transmitted is through cross-contamination while handling raw chicken meat during food preparation on a cutting-board. The unwashed cutting-board or utensil is subsequently used to prepare vegetables or other raw or lightly cooked foods. *Campylobacter* organisms from the raw meat can thus spread to the other previously non-contaminated foods (cross-contamination), through direct hand-to-mouth transfer from contaminated foods and to a lesser extent by the consumption of undercooked poultry meat. All these have been identified as important modes of

transmission (Tang *et al*., 2011). The risk posed by broiler meat to the total number of human campylobacteriosis cases accounts for between 50% to 80%, whereas the handling preparation and consumption of broiler meat may account for between 20% and 30% of all human chicken associated cases (EFSA, 2005).

Other foods associated with *Campylobacter* spp. infection include the drinking of unpasteurized milk, which can be contaminated through fecal contamination during milking and before the milk is pasteurized. Drinking water from untreated ground water supplies have been sources of infections in some reported outbreaks in Finland (Hänninen *et al*., 2003; Kuusi *et al*., 2005) as well. *Campylobacters* are frequently found in natural water bodies through the discharge of treated waste water and by fecal contamination from wild animals. In epidemiological studies swimming in natural water courses was shown to be a source of campylobacteriosis, mostly in children, during the summer time (Schönberg-Norio *et al*., 2004).

C. jejuni can remain dormant in water in a VBNC state (Rollins *et al*., 1986). This describes the situation that under unfavorable conditions, *C. jejuni* essentially remains dormant and cannot be easily recovered on artificial media.

2.7 The illness (campylobacteriosis)

Campylobacteriosis is a human illness caused by *Campylobacter* species. In industrialized countries, campylobacteriosis is characterized by sporadic infections throughout the population which is independent of age (Olson *et al*., 2008). However, in developing countries, the disease primarly occurs in infants due to high levels of exposure to the environment and acquired immunity of older children (Oberhelman & Taylor, 2000). Campylobacteriosis is very common illness in European countries, with a mean incidence of 45.6 confirmed cases per 100 000 inhabitants in 2009 (Table 3).

The species most commonly associated with human infection is *C. jejuni.* A minority of the infections are caused either by *C. coli* (up to 5% of the cases) or some other *Campylobacter* species.

Campylobacter jejuni is susceptible to low pH, and hence, the gastric environment is sufficient to kill most *Campylobacter* spp. (Black *et al*., 1988). An infective dose of *C. jejuni* is generally very low as studies have shown that consuming a small number of *Campylobacter* organisms, fewer than 500, can cause illness in humans (Robinson, 1981; Black *et al*., 1988). Anyone who has ingested the organism from contaminated food or water is at risk of becoming ill. In immunocompromised persons the risk of acquiring campylobacteriosis is higher still (Fernández-Cruz *et al*., 2010).

Symptoms usually appear two to five days after ingestion of the bacteria. Patients may experience mild to severe symptons, including fever, headache, abdominal cramps, diarrhea, with or without blood or fecal leukocytes present in the stool and nausea. In severe cases antimicrobial treatment is needed when symptoms are severe. Usually, infections are self-limiting and illness last for about a week, but relapses may occur in 5 to 10% of untreated patients. *C. jejuni* can occasionally spread to the bloodstream or cause life threatening infection in other parts of the body including infections such as pseudoappendicitis (Campbell *et al*., 2006), abdominal cavity, central nervous system, gallbladder, or urinary tract. *C. jejuni* infection can result in serious post-infectious sequelae, such as reactive arthritis, including Reiter's syndrome, or Hemolytic Uremic Syndrome (HUS), meningitis, recurrent colitis, acute cholecystitis, pancreatitis, cystitis, and rarely, approximately 1 in 1000 cases lead to a neurological disorder called Guillain-Barré syndrome (GBS), which manifests as a paralysis that may result in respiratory dysfunction severe neurological and even death (Murray *et al*., 2007; Nachamkin *et al*., 2008). Miller-Fischer syndrome is a rare variant of GBS that accounts for approximately 5% of GBS cases. Although most people who contract campylobacteriosis recover completely within 2 to 5 days, some *Campylobacter* infections can be fatal, for a 40 deaths out of 198 582 (0.02%) confirmed cases in the EU in 2009 (EFSA, 2009).

Table 3. Reported campylobacteriosis cases in humans 2005-2009 (adopted from The European Food Safety Authority, 2011).

Country	2005	2006	2007	2008	2009
	Confirmed cases (Confirmed cases/100 000)				
Austria	5065 (60.6)	5020 (60.1)	5821 (69.6)	4280 (51.2)	1 516 (18.1)
Belgium	6897 (64.5)	5771 (54.1)	5906 (55.4)	5111 (47.9)	5697 (53.4)
Bulgaria	-	0	38 (0.5)	19 (0.2)	26 (0.3)
Cyprus	-	2 (0.3)	17 (2.1)	23 (2.9)	37 (4.7)
Czech Rep.	30 268 (289.2)	22 571 (215.6)	24 137 (230.6)	20 067 (191.7)	20 259 (193.5)
Denmark	3677 (66.7)	3239 (58.7)	3868 (70.2)	3470 (62.9)	3353 (60.84)
Estonia	124 (9.2)	124 (9.2)	114 (8.5)	154 (11.5)	170 (12.7)
Finland	4002 (75.1)	3439 (64.6)	4107 (77.1)	4453 (83.6)	4050 (76)
France	2049 (3.2)	2675 (4.2)	3058 (4.8)	3424 (5.3)	3956 (6.1)
Germany	62 114 (75.7)	52 035 (63.4)	66 107 (80.6)	64 731 (78.9)	62 331 (76)
Hungary	8288 (82.6)	6807 (67.9)	5809 (57.9)	5516 (55)	6579 (65.6)
Ireland	1801 (40.5)	1810 (40.7)	1885 (42.3)	1752 (39.4)	1810 (40.7)
Italy	-	-	676 (1.1)	265 (0.4)	531 (0.9)
Lithuania	694 (20.7)	624 (18.6)	564 (16.8)	762 (22.7)	812 (24.2)
Luxembourg	194 (39.3)	285 (57.7)	345 (69.9)	439 (88.9)	551 (111.6)
Malta	91 (22)	54 (13.1)	91 (22)	77 (18.6)	132 (31.9)
Netherlands	3761 (44.1)	3186 (37.3)	3289 (38.5)	3341 (39.2)	3739 (43.6)
Poland	47 (0.1)	156 (0.4)	192 (0.5)	257 (0.7)	357 (0.9)
Romania	-	-	-	2 (0.01)	254 (1.2)
Slovakia	2204 (40.7)	2718 (50.2)	3380 (62.4)	3064 (56.6)	3813 (70.4)
Slovenia	-	944 (46.4)	1127 (55.4)	898 (44.2)	952 (46.8)
Spain	5513 (48.1)	5889 (51.4)	5055 (44.1)	5160 (45)	5106 (44.6)
Sweden	7692 (83.1)	7106 (76.8)	6078 (65.6)	5969 (64.5)	7178 (77.5)
U. K	52 686 (86.1)	52 134 (85.2)	57 815 (94.5)	55 609 (90.9)	65 043 (106.3)
EU Total	**195 426 (44.9)**	**175 561 (40.3)**	**200 507 (46.1)**	**190 566 (43.7)**	**198 582 (45.6)**

2.8 Modelling of bacterial survival in foods and water

In predictive microbiology, modeling of bacterial growth or survival is described as a function of environmental factors such as temperature, pH and water activity (McMeekin, 1993). Microbial models are mathematical expressions that quantify populations of microorganisms in a given food matrix or system as a function of relevant intrinsic or extrinsic variables (Whiting & Buchanan, 1993). There are several derived mathematical equations that describe the bacterial behavior under different external conditions.

2.8.1 Primary model

A primary model describes the microbial behavior (growth or survival) as a function of time under specific conditions. Quantities and parameters include colony forming units (CFUs), biomass, absorbance measurements, in addition to substrate levels or metabolic products depending on the model (Whiting, 1995). The most frequently used primary inactivation model is a log-linear model. It is favoured due to its simplicity. Nowadays there is strong evidence that the curves for bacterial cell survival are not log linear as the first order kinetic model entails (van Boekel, 2002). Among the various distribution functions that can describe monotonic survival curves, the Weibull distribution is probably the most convenient and flexible. It can be assumed that the inactivation patterns are due to biological response. There is no reason to accept that one model form would be universally valid for all microorganisms, substrates and physical conditions (Whiting, 1995).

2.8.1.1 The log-linear model

Traditionally microbial inactivation has been described to be analogous to chemical kinetics as a *first-order decay reaction* of the microbial population *N* (CFU/mL) during time *t* (Chick, 1908). In the linear model it is assumed that all cells in a population have equal sensitivity to external factors and that the death of an individual cell is dependent upon the random chance that a key molecule within it receives sufficient heat (Cole *et al*., 1993).

$$\frac{dN}{dt} = -kN \qquad (1)$$

integration of Eq (1) gives

$$\int_{N_0}^{N} \frac{dN}{N} = -\int_{t_0}^{t} K dt \qquad (2)$$

and therefore

$$ln\left(\frac{N}{N_0}\right) = -Kt \qquad (3)$$

or in decimal logarithms

$$log_{10}\left(\frac{N}{N_0}\right) = \frac{-K_{max}t}{ln10} \quad (4)$$

$$log_{10}(N) = log_{10}(N_0) - \frac{k_{max}t}{ln10} = log_{10}(N_0) - \frac{t}{D} \quad (5)$$

where *N* represents the microbial cell density, expressed in, [CFU/ml], for example, $N_{0,}$ the initial microbial cell density [CFU/ml], K_{max} [1/time unit] the first order inactivation constant and D [time unit] the decimal reduction time (the time required to achieve a 1-log reduction in the population) can be computed as $\ln(10)/k_{max.}$

The log-linear model (Eq. 5) is a single parameter model, which has the advantage of computational simplicity, in that it only requires the regression of survival data.

2.8.1.2 The Weibull model

In recent years nonlinearities in inactivation data have been addressed by several mathematical models (Anderson *et al*., 1996; Augustin *et al*., 1998; Baranyi & Pin, 2001; Peleg & Cole, 1998; Geeraerd *et al*., 2005). Among those models, considered to be the most important has probably been the use of the Weibull model. The Weibull distribution is considered to be the most convenient and flexible among the various distribution functions that describe monotonic survival curves. This distribution is named after Waloddi Weibull (1887-1979), a Swedish engineer and scientist, who was well-known for his work on the strength of materials and fatigue analysis (Weibull, 1939). The Weibull model is applicable to materials, structures and also to biological systems because it has an increasing failure rate and can describe wearing out processes. Nonthermal treatment studies are based on the hypothesis that the resistance to stress of a population follows a Weibull distribution (Peleg & Cole, 1998; Corradini & Peleg, 2003; Hajmeer *et al*., 2006). The Weibull model, when applied to describe microbial inactivation, is the cumulative form of the asymmetric

Weibull probability density function for the heat resistances of individual microbial cells. The cumulative distribution of the Weibull model can be applied in a variety of forms. For example in the logarithmic form (Eq 6).

$$log_{10}(N) = log_{10}(N_0) - \left(\frac{t}{\delta}\right)^p \quad (6)$$

Where p is a shape parameter, δ [time unit] is a scale parameter and can be explained as the time for the first decimal reduction when p = 1, in which case the Weibull model is capable of describing a wide range of inactivation phenomena, for which the log-linear is (p = 1). Convex curves are obtained for p > 1, whereas concave curves are described for p < 1.

2.8.2 Secondary models

Secondary models deal with the response of parameters that appear in primary modeling approaches as a function of one or more environmental conditions such as temperature or pH. The quality of the original data set is extremely important in generating the estimates. McDonald & Sun (1999) and Vereecken *et al*. (2000) presented a general overview of secondary model types. Nowadays, approaches that receive considerable attention for new developments are: (i) Bělehrádek type models (also referred to as Ratkowsky-type or square root models) (Ratkowsky *et al*., 1982), (ii) polynomial models (Gibson *et al*., 1988), (iii) cardinal values models (Rosso *et al*., 1995), and (iv) artificial neural network models (Hajmeer *et al*., 1997).

Great caution should be exercised to avoid extrapolation when using purely empirical secondary models, because the model could yield nonsensical results when applied outside the domain of the data from which the parameters were estimated. Most of these secondary models have little or no microbiological basis, which makes interpretation of some model parameters difficult and sometimes their performance are not stable.

2.8.3 Model validation

Evaluation of model performance usually involves the comparison of model predictions to analogous observations that were not used to develop the model. When a close similarity in mean square error (MSE) and correlation coefficient (r^2) values of the equations fitted to either dataset occurs this can be taken as an indication of the reliability of the model. F test can be also used in order to compare the goodness of fitness of parameters which have different number of parameters.

Other additional complementary measures of model performance namely bias factor and accuracy factor can be used to assess the validity of the model and are claimed to have the advantage of being interpretable (Ross, 1996). The bias factor is a multiplicative factor by which the model, usually over- or under- predicts the response time. The accuracy factor is also a simple multiplicative factor that indicates the spread of observation about the model's prediction.

3. AIMS OF THE STUDY

The aims of this PhD research were to identify association between *C. jejuni* isolates with hosts, characterize the survival of *C. jejuni* in different matrices (chicken meat and well water) in a wide range of temperature and to analyze the antimicrobial effect of some seasoning combinations in the survival of *C. jejuni* in chicken.

The specific aims were:

1. To apply new genetic markers associated either with amino acid metabolism (*ggt*), electron transfer (two oxidoreductase genes) and protease activity (a protease gene) so that a collection of *C. jejuni* isolates obtained from different hosts (human, chicken and bovine) can be studied to find the isolates association with these hosts (I).

2. To study survival of different *C. jejuni* strains in minced chicken meat, in marinated chicken meat treated with different seasonings and in well water (II, III, IV).

3. To model the survival of *C. jejuni* strains as a function of temperature in minced chicken meat and in well water (II, IV).

4. To test the effects of new combinations of food additives used as seasonings for the marination of chicken meat on decreasing Campylobacter CFUs (III)

5. To find if antibiotic resistance has effects on survival of *C. jejuni* strains survival (II, III, IV).

4. MATERIALS AND METHODS

4.1 Bacterial isolates (I-IV)

In **Study (I)** four new marker genes were identified in a total of 645 *C. jejuni* isolates. Of these 645 isolates, 131 were obtained from bovine fecal samples (Hakkinen *et al*., 2007), 205 from chicken cecal or chicken meat samples and 309 from human patients (Kärenlampi *et al*., 2007). Bacterial isolates that were used in Studies I-IV are shown in Table 4. MICs of ciprofloxacin, tetracycline, ampicillin and erythromycin upon the strains and their variants found in study I, then used in study II and IV are indicated in Table 5. All *Campylobacter* cultures were stored at – 70°C in skimmed milk that contained 15% glycerol. The isolates were recovered on Brucella agar (Oxoid Ltd., Basingstoke, Hampshire, England), which contained 5% horse blood, and which was incubated in a microaerobic atmosphere that contained (5% O_2, 10% CO_2, 85% N_2).

Table 4. Bacterial strains used in the studies I to IV.

Study	Species	Strains	Number of isolates	Isolation period	Origin
I	*C. jejuni*		131	(1996-2003)	Bovine
			205	(1996-2007)	Chicken
			309	(1996-2003)	Human
II	*C. jejuni*	49/7R			Poultry
		49/7RAT			
		49/7RATCIP32			
III	*C. jejuni*	1:1 mixture			
		(49/7R + ATCC33560)			
IV	*C. jejuni*	49/7R			Poultry
		49/7RAT			
		49/7RATCIP32			
		ATCC33560			Human(reference)
		ATCC33560CIP32			

Table 5. *Campylobacter jejuni* strains used in survival studies (II, III and IV) and the MICs for ciprofloxacin (CIP), tetracycline (TET), ampicillin (AMP) and erythromycin (ERY).

			*MIC (mg/L)			
Species/Source	Strain	Study	CIP	TET	AMP	ERY
C. jejuni/chicken	49/7R	II,III,IV	0.032	0.125	2	0.250
C. jejuni	49/7RAT	II,IV	0.5	4	16	0.250
C. jejuni	49/7RATCIP32	II,IV	128**	8	32†	16‡
C. jejuni/bovine	ATCC33560	III,IV	0.250	1	8	2
C. jejuni	ATCC33560CIP32	IV	128**	4	8	8

* MIC values from Hänninen and Hannula, 2007.
**Resistant to ciprofloxacin (breakpoint MIC 4 mg/L)
†Low-level resistance to ampicillin (breakpoint MIC 16 mg/L)
‡ Low-level resistance to erythromycin (breakpoint MIC 8 mg/L) (CLSI, 2009)
MIC values from Hänninen and Hannula, 2007.

4.2 Inoculation of meat and dug well water (II, III, IV)

The *C. jejuni* inoculums (**II**, **III** and **IV**) were prepared by spreading 100 µL from the frozen stock cultures onto Brucella agar plates (Oxoid Ltd, London, UK), which was supplemented with 5% horse or bovine blood, and the plates were incubated at 37°C under a microaerobic atmosphere (5% O_2, 10% CO_2, and 85% N_2) for 48h. A 1-µL loop of inoculum was transferred to a test tube that contained 5 mL of Brucella agar broth, and incubated at 37°C under microaerobic conditions for 24h and shaken at 150 r.p.m. Peptone water saline (0.1% peptone + 0.9% NaCl) was used for dilutions and for bacterial pellet resuspension.

In **study** (**II**), a *C. jejuni* inoculum was diluted to 1:100 and 1 mL (10^6 to 10^7) was inoculated in each minced chicken meat sample (10g portions). The inoculated *C. jejuni* was mixed in with the meat and the samples were stored in polyethylene bags at different temperatures (-20°C, -5°C, 4°C, 15°C and 25°C).

In **study (III)**, frozen chicken drumsticks that had been purchased from a local poultry processing plant were thawed and weighted. Each drumstick sample was inoculated with 1 mL of bacterial solution (10^6 – 10^7 CFU/ml) spread onto the surface of meat**.** After 15 min at 4°C, approximately 4 g of each seasoning combination dry mixture was spread evenly onto a drumstick and the drumsticks were packaged individually under a modified gas atmosphere (80% N_2, 20% CO_2) in sealed polyamide/polyethylene bags. The samples were stored at 4°C.

In **study** (**IV**) the inoculum culture of *C. jejuni* was centrifugated at 5600g for 15 min (Centrifuge 5810R, Eppendorf International, Hamburg, Germany). The supernatant was discarded and the bacterial pellets were resuspended in 5 mL peptone water and then diluted (1mL + 99mL of PPS). A 1 mL volume of the diluted inoculums was transferred to a sterile glass bottle that containing 99 mL of well water that had been collected from a dug well located in a rural area in Finland (pH ranged from 6.7 to 6.8 and heterotrophic counts < 5 CFU/ml).

4.3 Sampling and cultivation

Study (II). Samples of each storage temperature treatment at the time of inoculation, after 4h, then 1, 2, 3, 4, 5, 6, 7, 11, 14, 21, 28 and 56 days were taken (Table 6). Experiments that evaluated survival at each storage temperature were repeated twice. First time with duplicate samples, and second time with triplicate samples. At each sampling interval, frozen samples were thawed at room temperature for 20 min and 10 g quantities were subsequently diluted 1:10 in 0.9% peptone saline water. These were subsequently mixed for 20 s and spread on mCCDA (Oxoid) plates, incubated under microaerobic conditions for at 37°C for 48 h. CFUs/ml were subsequently determined.

Study (III). At each time point, at inoculum time, after 15 min, then 1 h, 1 day, and at 7 days, 99 ml of 0.1% peptone saline water was poured into a sample bag and mixed for 20 s. The suspension was serially diluted in peptone saline water, and a 0.1 ml volume inoculum was spread onto duplicate mCCDA plates. The mCCDA plates were then incubated under microaerobic conditions at 37°C for 48 h. At least two replicates of each experiment were performed using triplicate samples for each time point, and the counts in CFU/g were determined.

Study (IV). The inoculated well water was stored in bottles, which were kept in the dark at 4°C for a maximum of 70 days, at 10°C for 30 days, at 15°C for 20 days, at 20°C for 9 days, and at 25°C for 6 days (Table 6). A 1 ml sample was taken and 10-fold dilutions were made in PPS at T_0, 4 h and then 1, 2, 3, 6, 8, 10, 14, and 18 days and thereafter weekly until day 70. A 0.1-ml volume of appropriate dilutions was spread on modified cefoperazone charcoal deoxycholate agar (mCCDA, Oxoid) plates. The CFUs were enumerated after being incubated under a microaerobic atmosphere at 37°C for 48 h. The means and standard deviations of every inoculated well-water bottle.

Table 6. Storage periods and temperature regimes for (days) the different *C. jejuni* studies (II, III, IV).

Study	Strains	-20°C	-5°C	4°C	10°C	15°C	20°C	25°C
II	49/7R	56	56	11	-	6	-	6
	49/7RAT	56	56	9	-	6	-	6
	49/7RATCIP32	56	56	9	-	6	-	6
III	1:1 mixture 49/7R and ATCC33560	-	-	7	-	-	-	-
IV	49/7R	-	-	70	30	20	9	6
	49/7RAT	-	-	70	30	20	9	6
	49/7RATCIP32	-	-	70	30	20	9	6
	ATCC 33560	-	-	70	30	20	9	6
	ATCC 33560CIP32	-	-	70	30	20	9	6

4.4 PCR of genetic marker genes (I)

In order to investigate host association of *C. jejuni* isolates from humans, bovine and chicken, we tested four new genetic markers: *ggt* (the γ-glutamyl transpeptidase gene); (*Cju34*), a subunit of the putative tripartite anaerobic dimethyl sulfoxide oxidoreductase; *Cj1585c* (that codes for a putative oxidoreductase); and *Cjj81176-1371* (a putative serine protease gene) and tested presence/absence of these markers genes by PCR (Hofreuter *et al*., 2006). The PCR primers that were designed for the amplification of the fragments are shown in Table 7.

Table 7. Primers used in PCR of the fragment of the four marker genes.

Marker gene (product)	Primer sequence		Product size (bp)
	Forward	Reverse	
ggt	AGCTGCTGGAGTACCAGGAA	TTTTAGCCATATCGCCTGCT	339
Cju34	GATAGGGCATTGCGATGAGT	CTTGCTAGCCCAATCAGGAG	238
Cj1585c	TGTTGTGGGTTTGCTGGATA	TTGCTTCACTGCATTCATCC	202
Cjj81176-1367/1371	TGCAAAGCAGGGCTAAGAAT	TTATGGAGCTGGGGTGTTTC	318

The PCR conditions for these four marker genes were as follows: the reaction mixture (50 µl) consisted of 0.2 mM each dNTP (Finnzymes, Espoo, Finland), 0.2 µM PCR primer (Oligomer, Helsinki, Finland), 1 unit of Dynazyme polymerase (Finnzymes) and approximately 50 ng of template DNA. The cycling conditions were: denaturation at 95°C for 30s, annealing at 55°C for 45s, and extension at 72°C for 1 min for 30 cycles in total. The *C. jejuni* Strain 81-176 was used as a positive control and *C. jejuni* NCTC 11168 as a negative control.

4.5 Effect of seasoning combinations on *C. jejuni* counts on chicken meat (III)

The composition of the seasoning combinations that were added as a dry mixture of compounds onto the surface of chicken samples differed mainly in the quantity of added sodium lactate content (+/-) and low or high molecular weight fraction of potato proteins (+/-) as a proportion of the total weight (Table 8). These six seasoning combinations were prepared specifically for this study. All of the combination ingredients have been marketed all over the world individually or as mixtures accepted as food additives and ingredients.

Table 8. Chemical composition of the seasoning combinations for treatment A, B, C, D, E, and F.

	Seasoning treatments					
Components	A	B	C	D	E	F
Maltodextrin	x	x	x	x	x	x
Potato fiber	x	x	x	x	x	x
Monosodium glutamate (E621)	x	x	x	x	x	x
Sodium metabisulfite (E223)	x	x	x	x	x	x
Ethylenediaminetetraacetic acid (E385)	x	x	x	x	x	x
Xantham gum (E415)	x	x	x	x	x	x
Konjac gum (E425)	x	x	x	x	x	x
Methyl cellulose (E461)	x	x	x	x	x	x
Modified potato starch (E1424)	x	x	x	x	x	x
Paprika	x	x	x	x	x	x
Coriander	x	x	x	x	x	x
Black pepper	x	x	x	x	x	x
White pepper	x	x	x	x	x	x
Garlic	x	x	x	x	x	x
Capsium nutmeg	x	x	x	x	x	x
Celery	x	x	x	x	x	x
Sodium chloride (16%)	x	x	x	x	x	x
Sodium lactate (24%) (E325)	-	x	-	x	-	x
Potato protein high molecular weight fraction (4.8%)	-	-	x	x	-	-
Potato protein low molecular weight fraction (4.8%)	-	-	-	-	x	x

4.6 Data analysis

4.6.1 Data analysis for model prediction (II, III, IV)

For data analyses and calculations Microsoft® Excel 2003 (II) and Microsoft® Excel 2007 (III, IV) were used. GInaFiT software as used described by Geeraerd *et al*., (2005) was used to identify appropriate survival models that fit the dataset by least square regression and a logarithmic form of the Weibull model was selected in order to build a predictive model.

4.6.2 Primary model (II, III, IV)

Empirical data were fitted using GInaFiT to 10 different models. The best fitting and simple logarithmic form of the Weibull model (Eq 6) was selected to build the primary model.

$$log_{10}(N) = log_{10}(N_0) - \left(\frac{t}{\delta}\right)^p \quad (6)$$

4.6.3 Secondary model (II, IV)

In order to build a secondary model, Weibull parameters δ and p that were obtained in the primary model were fitted as a function of the temperature for different secondary models. It was found that a third order polynomial order which showed the highest determination coefficient and the best fit. In **study II** the standard deviation of experimental data was higher than in **study IV**. In study II we did not calculate $\delta = \delta$ (T) and $p = p$ (T) directly, but instead log (δ (T)) and log (p (T)) in order to improve the goodness of fit. Parameters $\delta = \delta$ (T) and $p = p$ (T) were obtained using the antilogarithm of log (δ (T)) and log (p (T)). In **study IV** the logarithmic form was not needed, and secondary model parameters were modeled as $\delta = \delta$ (T) and $p = p$ (T).

Secondary model parameters were substituted in predicting model (Eq 7).

$$log_{10}(N) = log_{10}(N_0) - \left(\frac{t}{\delta(T)}\right)^{p(T)} \quad (7)$$

4.6.4 Model validation (II, IV)

In **study II**, predictions were compared with actual survival values of *C. jejuni* from the ComBase database (www.wyndmoor.arserrc.gov/combase) in order to assess the performance of the predictive model.

In **study IV**, the predictive model was validated by comparison of the predicted survival of *C. jejuni* with independent data provided by Cook & Bolster (2007).

4.6.5 Death rate calculation (III)

In **study III** we analyzed the effectiveness of different seasoning combinations on drumsticks on the decline of *C. jejuni* counts by calculating the death rate at each sampling point. From study II we knew that the Weibull model optimally describes the survival of these microorganisms in minced chicken meat. We used (Eq 7) for describing the survival of *C. jejuni*. Equation 7 was rearranged to describe relative reduction S (Eq 8). Model parameters δ and p depend on the temperature, but as we were working only at a single temperature (4°C), those parameters could be derivates of time as if they had been constant values. Equation 9 shows the death rate as a function of the stored time, and model parameters δ and p.

$$log_{10}S(t) = log_{10}\left(\frac{N(t)}{N_0}\right) = -\left(\frac{t}{\delta}\right)^{p} \quad (8)$$

$$\frac{dlog_{10}S(t)}{dt} = -\frac{p}{\partial^{p}}t^{(p-1)} \quad (9)$$

4.6.6 Statistical analysis

Statistical analyses were performed using SPSS 15 software. In **study I**, comparisons for similarities in the frequencies of marker genes between the isolates obtained from different hosts was performed using the $\chi 2$ test. In addition, we used the paired two-tailed Student's t-test for the analysis of host association for the combined set of four genes. A $P < 0.05$ was considered significant. Analysis of the differences for the time required for 1-log reduction (**II, IV**) and 4-log reduction (**IV**) among native strains and their antimicrobial resistance variants were done with t-test test with a significance level of $P < 0.05$. F-test was used to compare the goodness of fitting between the log-linear model and the Weibull model.

5. RESULTS

5.1 Host association of *C. jejuni* strains (I)

In **study I**, PCR of the presence or absence of four selected marker genes was shown to divide the strains within each group. Twelve PCR products for each gene fragment were sequenced. The sequences of each gene were shown to be highly conserved (95.5 to 100% similarity within each gene) because only a few nucleotide positions (from 2 to 9) were found to be polymorphic.

In Figure 1 the frequencies of the genes and the results of the paired two-tailed *t* test for the significance of the frequencies of the combined four genes are shown. These results indicated significant associations of bovine and chicken isolates with their host source ($P < 0.05$). An high similarity was found between chicken and human isolates ($P = 0.9949$). However the bovine isolates differed significantly ($P < 0.05$) from human and chicken isolates.

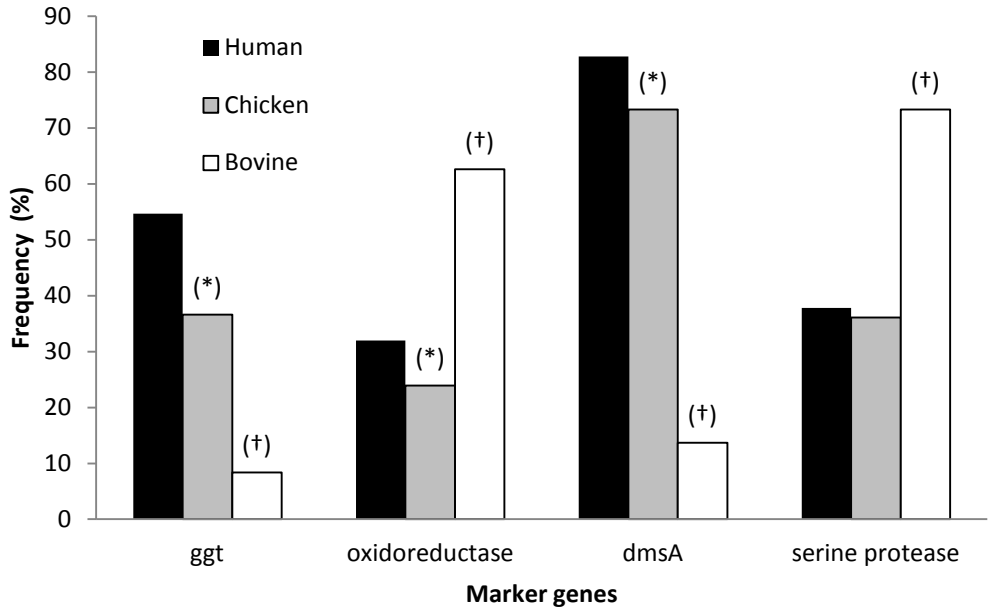

Figure 1. Frequencies of the four marker genes *ggt, Cj*1585c, *dmsA* (Cju34), and *Cjj*81176-1371 in 645 human, chicken, and cattle *C. jejuni* isolates. (*) $P < 0.05$ represents significant difference between human and chicken. (†) $P < 0.05$ represents significance differences between human and bovine isolates. Significance of the frequency of the combined four genes by paired two-tailed t test, (P (Hu-Ch) = 0.9949; (Ch-Bo) = 0.0087 and (Hu-Bo) = 0.0122).

The frequency of the *ggt*-positive human and chicken isolates was high but very low for the bovine isolates. The frequencies of serine protease positive strains between bovine and human isolates were significantly different. Human and chicken isolates had approximately similar frequencies of serine protease positive strains.

The *Cj1585c*-type oxidoreductase was present among the isolates from cattle more frequently than those obtained from chickens or humans. In contrast, the *dmsA* (*Cju34*) was more present more often in human and chicken isolates than in those obtained from bovines. The frequency of serine protease in bovine isolates was around 73.3% whereas in isolates from human and chicken they were 37.8% and 36.1% respectively (Figure 1).

5.2 Trends of the gene marker frequencies for human and chicken according to the year of isolation (I)

In Figure 2 the annual frequencies of the four marker genes for isolates from humans are presented. The analysis of the annual frequencies of the four genes combined was found to be similar for 1996 and 2002, and also for 2002 and 2003. However, the frequency of the genes differed between 1996 and 2003.

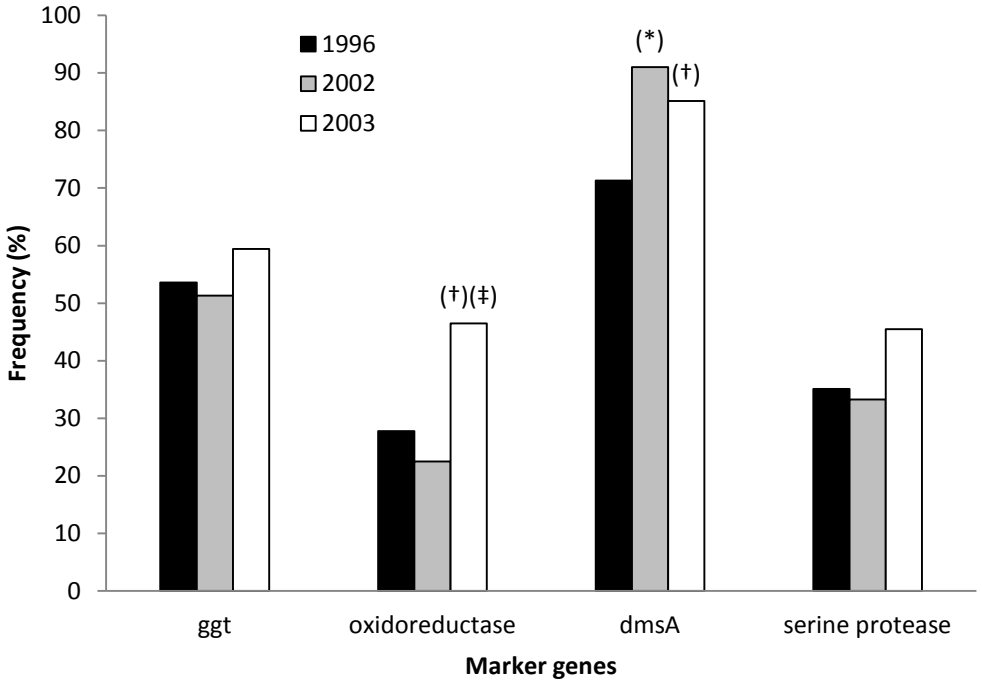

Figure 2. Frequencies of the four marker genes *ggt, cj*1585*c*, *dmsA* (*cju*34), and cjj81176-1371 in 309 human *C. jejuni* isolates distributed according to the year of their isolation (1996, 2002 and 2003). (*) $P < 0.05$ represents significant differences between 1996 and 2002. (†) $P < 0.05$ represents significant differences between 1996 and 2003. (‡) $P < 0.05$ represents significant differences between 2002 and 2003.

The annual frequencies of the four marker genes for chicken isolates are presented in Figure 3. The analysis of the annual frequencies of the four combined genes indicated that chicken isolates were similar in all the compared time periods.

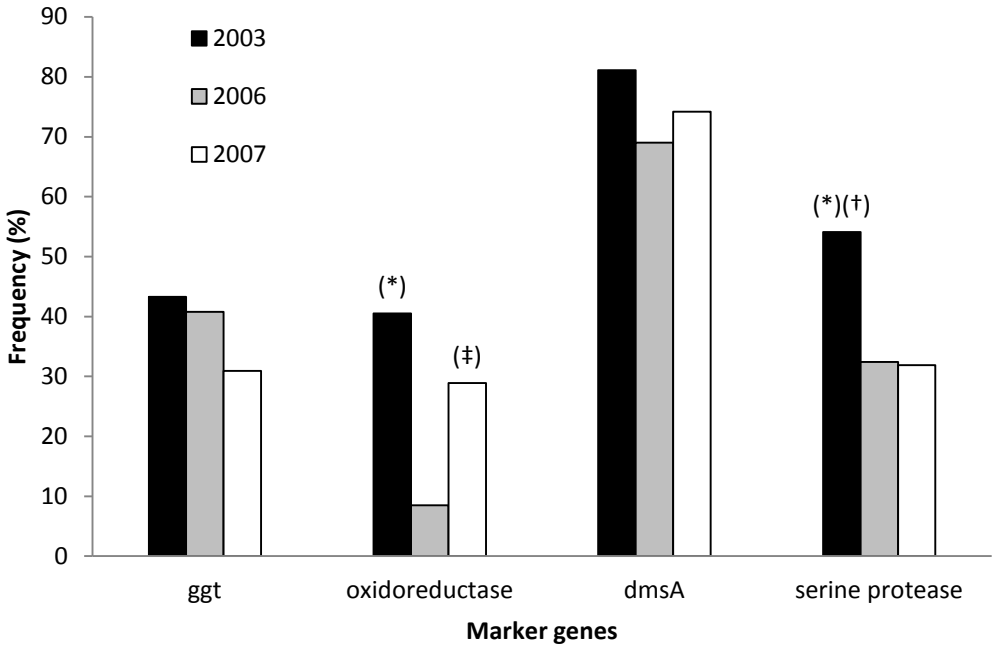

Figure 3. Frequencies of the four marker genes *ggt, cj*1585*c*, *dmsA* (*cju*34), and *cjj*81176-1371 in 205 chicken *C. jejuni* isolates distributed according to the year of their isolation. (*) $P < 0.05$ represents significant differences between 2003 and 2006. (†) $P < 0.05$ represents significant differences between 2003 and 2007. (‡) $P < 0.05$ represents significant difference between 2006 and 2007.

5.3 Primary model for *C. jejuni* survival (II, IV)

In order to select a primary model, we compared the data fit of the log linear model and the Weibull model (Tables 9A and 9B).

Table 9A. Statistical comparison of data fitting among the log linear and the Weibull model for *C. jejuni* 49/7R, 49/7RAT and 49/7RATCIP32.

		Minced chicken meat				Well water			
Strain	T(°C)	SS_{linear}	$SS_{Weibull}$	Ratio F	P_{value}	SS_{linear}	$SS_{Weibull}$	Ratio F	P_{value}
A*	-20	0.066	0.001	845	<0.05†	-	-	-	-
	-5	0.098	0.010	114.4	<0.05†	-	-	-	-
	4	0.004	0.004	0.84	0.37	0.057	0.046	3.9	0.06
	10	-	-	-	-	0.128	0.092	3.9	0.08
	15	0.009	0.003	26	<0.05†	0.408	0.023	115.1	<0.05†
	20	-	-	-	-	0.497	0.002	1442.6	<0.05†
	25	0.006	0.002	26	<0.05†	0.382	0.044	30.9	<0.05†
B**	-20	0.076	0.005	184.6	<0.05†	-	-	-	-
	-5	0.021	0.002	123.5	<0.05†	-	-	-	-
	4	0.004	0.002	13	<0.05†	0.022	0.012	10.9	<0.05†
	10	-	-	-	-	0.015	0.004	19.5	<0.05†
	15	0.005	0.001	52	<0.05†	0.088	0.056	3.3	0.12
	20	-	-	-	-	0.113	0.079	2.5	0.16
	25	0.007	0.003	17.3	<0.05†	0.021	0.004	20.2	<0.05†
C***	-20	0.094	0.004	292.5	<0.05†	-	-	-	-
	-5	0.083	0.003	346.7	<0.05†	-	-	-	-
	4	0.092	0.001	1183	<0.05†	0.124	0.010	159.5	<0.05†
	10	-	-	-	-	0.022	0.019	1.2	0.29
	15	0.002	0.001	13	<0.05†	0.312	0.110	13.2	<0.05†
	20	-	-	-	-	0.266	0.116	7.7	<0.05†
	25	0.022	0.001	273	<0.05†	0.034	0.028	1.3	0.30

†P <0.05 data fit significantly better to the Weibull than to log-linear model.
*49/7R.
**49/7RAT.
***49/7RATCIP32.

Table 9B. Statistical comparison of data fitting among the log linear and the Weibull model for *C. jejuni* ATCC 33560 and ATCC 33560CIP32.

		Minced chicken meat				Well water			
Strain	T(°C)	SS_{linear}	$SS_{Weibull}$	Ratio F	P_{value}	SS_{linear}	$SS_{Weibull}$	Ratio F	P_{value}
D*	-20	-	-	-	-	-	-	-	-
	-5	-	-	-	-	-	-	-	-
	4	-	-	-	-	0.050	0.038	4.6	<0.05†
	10	-	-	-	-	0.099	0.087	1.5	0.25
	15	-	-	-	-	0.010	0.010	0.5	0.48
	20	-	-	-	-	0.386	0.161	5.5	0.08
	25	-	-	-	-	0.099	0.097	0.1	0.79
E**	-20	-	-	-	-	-	-	-	-
	-5	-	-	-	-	-	-	-	-
	4	-	-	-	-	0.099	0.097	1.9	0.18
	10	-	-	-	-	0.190	0.089	17.1	<0.05†
	15	-	-	-	-	0.130	0.019	68.6	<0.05†
	20	-	-	-	-	0.184	0.009	122.3	<0.05†
	25	-	-	-	-	0.136	0.121	0.5	0.52

† $P < 0.05$ data fit significantly better to the Weibull than to log-linear model.
* ATCC 33560.
** ATCC 33560CIP32.

Survival data for the *C. jejuni* strain 49/7R and its resistant variants 49/7RAT and 49/7RATCIP32 in minced chicken meat and in well water were fitted to the Weibull model. Data of *C. jejuni* ATCC 33560 and ATCC 33560CIP32 survival in well water were also fitted to the Weibull model as well because it was observed that data fit significantle better ($P < 0.05$) to the Weibull model than the log linear in 27 out 40 cases studied (Tables 9A and 9B).

The parameters of the Weibull model δ and p and the goodness of fit values are shown with statistical indices RMSE and R^2_{adj} (Table 10).

Table 10. Parameter estimates and statistical comparison analysis of the fitting to the Weibull model for *C. jejuni* in minced chicken meat and in well water data.

		Matrices for modelling							
		Minced meat chicken				Well water			
Strain	**T(°C)**	***δ***	***p***	**R^2_{adj}**	**RMSE**	***δ***	***p***	**R^2_{adj}**	**RMSE**
49/7R	-20	3.32	0.27	0.996	0.034	-	-	-	-
	-5	6.28	0.64	0.995	0.100	-	-	-	-
	4	11.07	0.85	0.963	0.065	17.90	1.24	0.980	0.214
	10	-	-	-	-	8.60	1.51	0.951	0.304
	15	5.47	0.61	0.982	0.052	5.68	2.75	0.992	0.153
	20	-	-	-	-	4.42	2.68	0.999	0.049
	25	2.23	0.73	0.993	0.044	3.01	2.18	0.984	0.209
Mean				**0.986**	**0.059**			**0.981**	**0.186**
49/7RAT	-20	5.26	0.32	0.987	0.068	-	-	-	-
	-5	7.14	0.62	0.997	0.042	-	-	-	-
	4	18.48	0.83	0.997	0.011	13.20	1.17	0.995	0.108
	10	-	-	-	-	4.28	1.16	0.998	0.066
	15	12.25	0.54	0.997	0.012	1.28	0.78	0.982	0.237
	20	-	-	-	-	1.16	0.76	0.974	0.282
	25	2.61	0.66	0.986	0.053	1.51	1.18	0.998	0.064
Mean				**0.993**	**0.037**			**0.989**	**0.151**
49/7RATCIP32	-20	8.79	0.40	0.992	0.062	-	-	-	-
	-5	10.08	0.42	0.993	0.055	-	-	-	-
	4	27.86	0.64	0.996	0.011	12.56	1.60	0.997	0.100
	10	-	-	-	-	3.00	0.98	0.993	0.139
	15	12.99	0.67	0.994	0.017	3.07	1.94	0.966	0.332
	20	-	-	-	-	2.36	1.74	0.968	0.341
	25	3.30	0.58	0.994	0.041	1.35	1.13	0.993	0.168
Mean				**0.994**	**0.037**			**0.983**	**0.216**
ATCC33560	4	-	-	-	-	15.84	1.21	0.987	0.195
	10	-	-	-	-	6.67	1.04	0.973	0.295
	15	-	-	-	-	4.49	1.07	0.997	0.099
	20	-	-	-	-	1.74	1.64	0.966	0.402
	25	-	-	-	-	1.14	1.17	0.980	0.312
Mean								**0.981**	**0.261**
ATCC33560CIP32	4	-	-	-	-	13.81	1.00	0.986	0.205
	10	-	-	-	-	11.76	1.67	0.970	0.298
	15	-	-	-	-	7.1	1.62	0.995	0.139
	20	-	-	-	-	3.2	1.90	0.997	0.093
	25	-	-	-	-	1.23	1.09	0.973	0.348
Mean								**0.984**	**0.216**

The mean values of R^2_{adj} for the Weibull model ranged from 0.986 to 0.994 in the minced chicken meat matrix and from 0.981 to 0.989 in well water. The mean values of RMSE for the Weibull model were within the interval of 0.037 to 0.059 for the minced chicken meat whereas well water RMSE values ranged from 0.151 to 0.261. Both statistical indices indicated that the Weibull model fitted the empirical data optimally. The δ parameter in the Weibull model also indicates the time required (in days) to obtain a reduction of *C. jejuni* counts of 1-log CFU/g in minced meat chicken and the time needed (in days) for a reduction of 1-log CFU/ml of *C.jejuni* of well water.

5.4 Secondary model for *C. jejuni* survival (II, IV)

The secondary model parameter δ was modeled as a function of the temperature using a third order polynomial, with a coefficient of determination that ranged from 0.905 to 0.963 in minced chicken meat and from 0.987 to 0.999 for well water (Table 11).

Table 11. Estimates for parameter δ, in secondary model for *C. jejuni* in minced chicken meat and in well water.

	Minced chicken meat $\log\delta(T)=C_3T^3+C_2T^2+C_1T+C_0$					Water from dug well $\delta(T)=W_3T^3+W_2T^2+W_1T+W_0$				
Strains	C_3	C_2	C_1	C_0	R^2	W_3	W_2	W_1	W_0	R^2
49/7R	-2.10^{-5}	-9.10^{-4}	9.10^{-3}	0.92	0.937	0.003	0.17	-3.47	29.30	0.999
49/7RAT	-6.10^{-5}	-7.10^{-4}	3.10^{-2}	1.07	0.963	-0.002	0.14	-3.18	23.56	0.999
49/7RATCIP32	-5.10^{-5}	-7.10^{-4}	2.10^{-2}	1.22	0.905	-0.005	0.26	-4.42	26.21	0.987
ATCC33560	-	-	-	-	-	-0.002	0.13	-2.93	25.54	0.993
ATCC33560CIP32	-	-	-	-	-	0.003	-0.13	1.04	11.63	0.999

The shape parameter p was also fitted as a function of temperature by a third order polynomial with coefficients of determination that ranged from 0.880 to 0.953 in minced chicken meat and from 0.741 to 0.972 for well water (Table 12).

Table 12. Estimates for parameter p, in secondary model for *C.jejuni* in minced chicken meat and in water from well water.

	Minced chicken meat $\log p(T)=C_3T^3+C_2T^2+C_1T+C_0$					Dug well water $p(T)=W_3T^3+W_2T^2+W_1T+W_0$				
Strains	C_3	C_2	C_1	C_0	R^2	W_3	W_2	W_1	W_0	R^2
49/7R	2.10^{-5}	-6.10^{-4}	1.10^{-3}	-0.13	0.953	0.0010	0.03	-0.27	1.76	0.923
49/7RAT	2.10^{-5}	-5.10^{-4}	-1.10^{-2}	-0.15	0.880	0.0005	-0.02	0.18	0.71	0.972
49/7RATCIP32	-2.10^{-5}	7.10^{-6}	1.10^{-2}	-0.29	0.931	-0.0010	0.05	-0.59	3.18	0.768
ATCC33560	-	-	-	-	-	-0.0007	0.03	-0.37	2.27	0.741
ATCC33560CIP32	-	-	-	-	-	-0.0003	0.005	0.05	0.74	0.867

5.5 Validation of model performance (II, IV)

In **study II** our model was validated comparing it against external data reported by Bhaduri and Cottrell, (2004) and from (www.wyndmoor.arserrc.gov/combase) and for **study IV** we validated our model against the external data published by Cook & Bolster (2007) (Table 13). In all cases bias and accuracy factors were very close to one, and the mean values for empirical points that fell inside the acceptable prediction

zone were in excess of 70%, which indicated that the model was suitable for predicting inactivation curves of *C. jejuni*.

Table 13. Validation performance results.

		Matrix								
Strain		**Chicken**					**Water**			
		[a]Ref.	[b]n	[c]B.F	[d]A.F	[e]APZ	[a]Ref.	[b]n	[c]B.F	[d]A.F	[e]APZ
49/7R		[f]BC	5	0.98	1.09	100	[h]CB	7	0.77	1.74	**71**
		[g]M3_CJ	5	1.15	1.15	60					
		[g]M26_CJ	9	1.09	1.09	66.6					
		[g]M27_CJ	11	0.96	1.06	72.7					
		[g]M29_CJ	10	0.98	1.05	100					
		[g]M30_CJ	11	0.92	1.08	54.5					
		[g]M31_CJ	12	0.93	1.07	58.3					
	subtotal					**71.4**					
49/7RAT		[f]BC	5	0.99	1.04	100	[h]CB	7	1.12	1.61	**71**
		[g]M3_CJ	5	1.16	1.16	60					
		[g]M26_CJ	9	1.13	1.13	66.6					
		[g]M27_CJ	11	1.12	1.15	72.7					
		[g]M29_CJ	10	1.02	1.04	90					
		[g]M30_CJ	11	0.97	1.04	90.9					
		[g]M31_CJ	12	0.97	1.03	91.6					
	subtotal					**82.5**					
49/7RATCIP32		[f]BC	5	1.02	1.18	100	[h]CB	7	0.94	1.67	**100**
		[g]M3_CJ	5	1.16	1.16	60					
		[g]M26_CJ	9	1.14	1.14	66.6					
		[g]M27_CJ	11	1.16	1.19	54.5					
		[g]M29_CJ	10	1.03	1.05	90					
		[g]M30_CJ	11	0.98	1.03	100					
		[g]M31_CJ	12	0.98	1.03	100					
	subtotal					**82.5**					
ATCC33560							[h]CB	7	1.16	1.85	**71**
ATCC33560CIP32							[h]CB	7	1.02	1.73	**71**

[a] Ref. is the external reference.
[b] Is the number of samples in the dataset.
[c] B.F is the bias factor
[d] A.F is the Accuracy factor
[e] Acceptable prediction zone.
[f] from Bhaduri and Cottrell, 2004.
[g] Record key from *ComBase*.
[h] from Cook and Bolster, 2007

5.6 Decline of *C. jejuni* counts on chicken meat treated with different seasoning combinations (III)

The reduction of *C. jejuni* counts in the control (untreated) samples after seven days was 0.21±0.02 log CFU/g. The effect of seasoning combinations A and B were compared and the results are presented in Figure 4. Seasoning combination B was identical to A, except that it also contained sodium lactate. The mean count decline with seasoning combination A was 1.02±0.07 log CFU/g and 1.06±0.08 log CFU/g with combination B after one day of storage. There was no marked decline during the following six days.

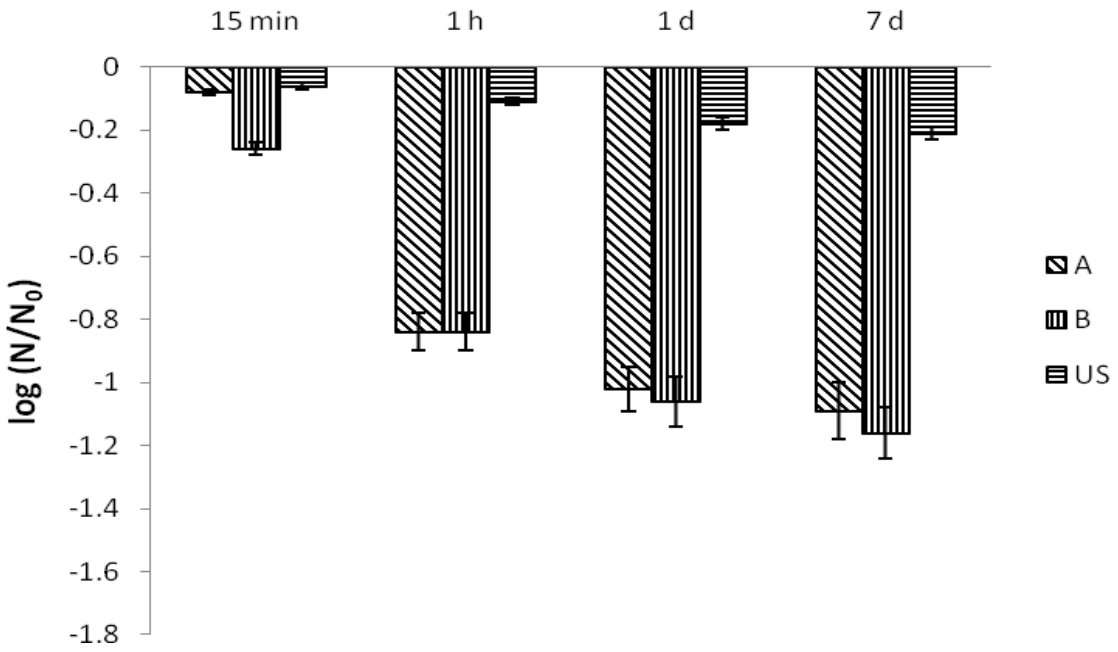

Figure 4. Reduction of mean log CFU/g of a 1:1 mixture of *C. jejuni* strains ATCC33560 and 49/7R on chicken drumsticks packaged in a modified gas atmosphere (80% N_2 and 20% CO_2) treated with seasoning combinations A or B then stored at 4°C and sampled after 15 min, 1h, 1 day and 7 days. The reduction is expressed as log (N/N_0), where N_0 is count at the beginning and N is count at the indicated sampling time. The untreated samples are marked as US.

The total mean decline in log CFU/g during the seven days of storage was greater in those samples treated with a seasoning combination that contained a potato protein fraction and sodium lactate (D and F) (Figure 5 and 6) than in samples without these compounds (C and E) (Figure 5 and 6). The mean total reductions in *C. jejuni* counts in combinations D and F were 1.52±0.12 and 1.66±0.14 log CFU/g, respectively, whereas the mean decline in groups C and E were 1.09±0.08 and 1.10±0.09 log CFU/g, respectively (Figures 5 and 6)**.**

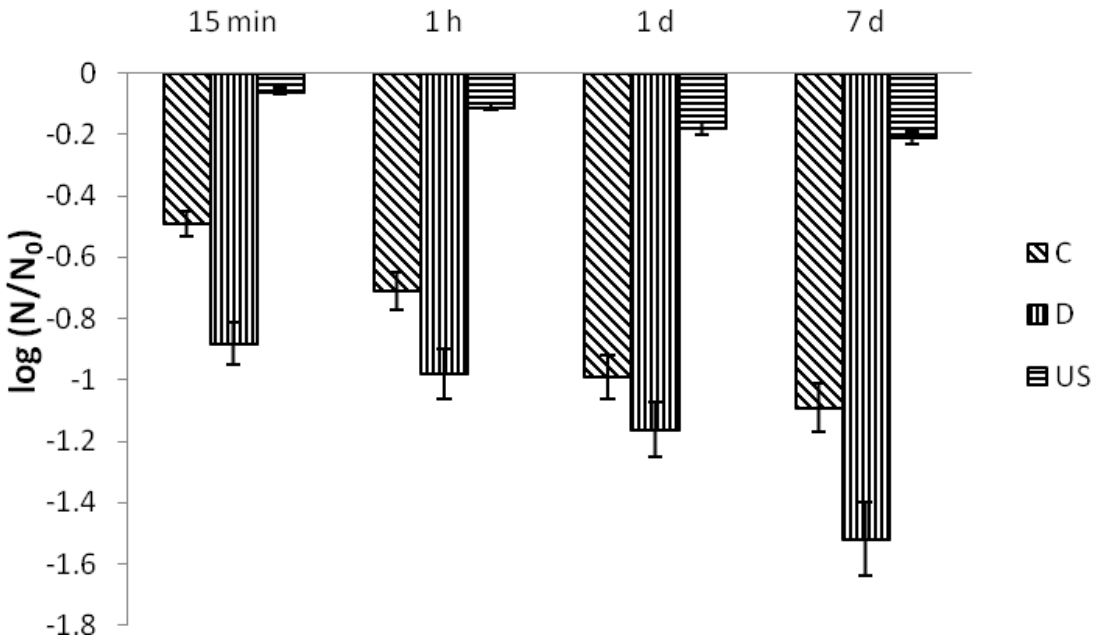

Figure 5. Reduction in mean log CFU/g of a 1:1 mixture of *C. jejuni* strains ATCC33560 and 49/7R on chicken drumsticks packaged in a modified gas atmosphere (80% N_2 and 20% CO_2) treated with seasoning combinations C or D stored at 4°C and sampled after 15 min, 1 h, 1 day and 7 days. The reduction is expressed as log (N/N_0), where N_0 is count at the beginning and N is count at the indicated sampling time. The untreated samples are marked as US.

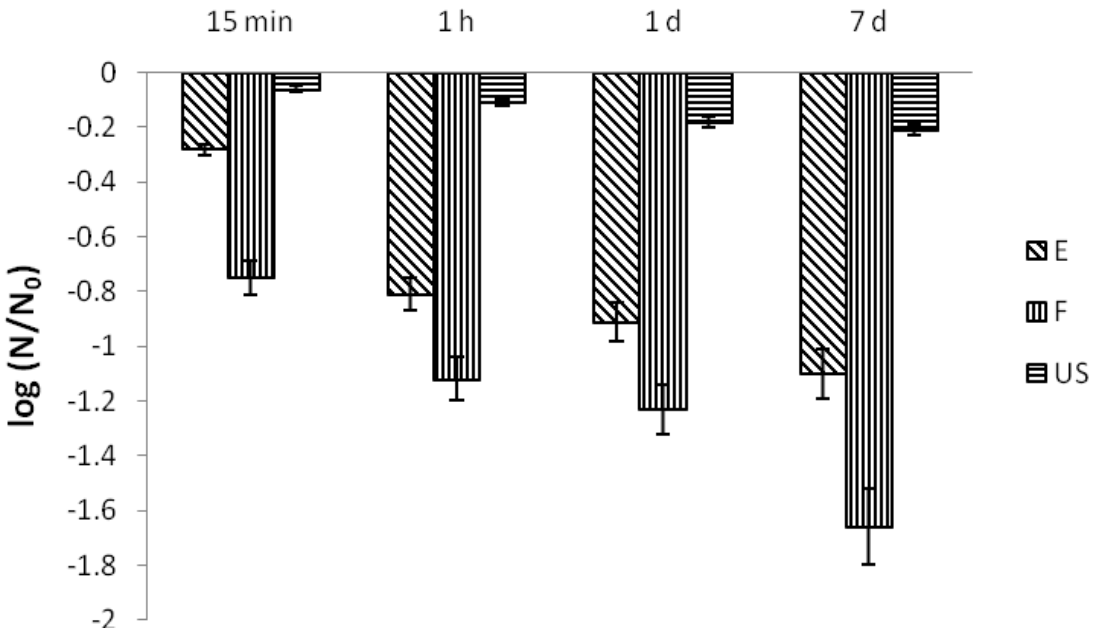

Figure 6. Reductions of mean log CFU/g of a 1:1 mixture of *C. jejuni* strains ATCC33560 and 49/7R on chicken drumsticks packaged in a modified gas atmosphere (80% N_2 and 20% CO_2), treated with seasoning combinations E or F and sampled stored at 4°C and sampled after 15 min, 1 h, 1 day and 7 days. The reduction is expressed as log (N/N_0), where N_0 is count at the beginning and N is count at the indicated sampling time. The untreated samples are marked as US.

5.7 Death rates of *C. jejuni* on chicken meat treated with different seasoning combinations (III)

We calculated that the greatest death rate occurred at the beginning of the experiment using (Eq 9). The seasoning combination F had the highest death rate after 15 minutes at a rate of -0.34 log CFU/g/h (Figure 7).

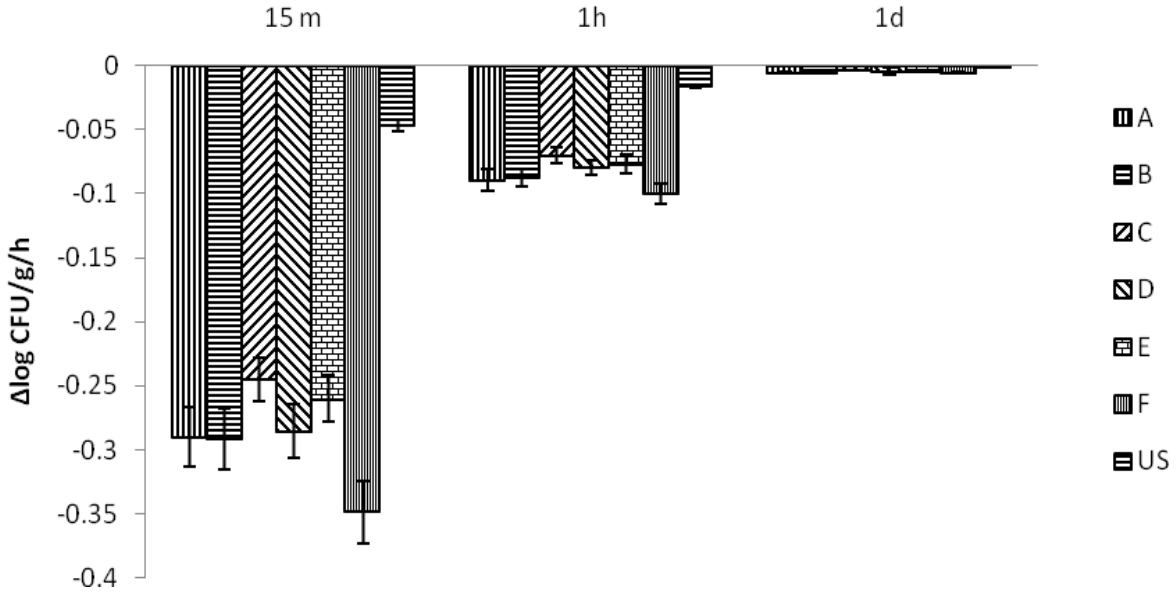

Figure 7. Death rates of a 1:1 mixture of *C. jejuni* strains ATCC33560 and 49/7R at different sampling times (15 min, 1h and 1 day) on chicken drumsticks treated with seasoning combinations A to F; untreated samples are marked as US.

6. DISCUSSION

6.1 Host association of *C. jejuni* strains (I)

C. jejuni colonizes a broad range of hosts, compromising both domestic and wild animals that offer extensive opportunities to evolve in their hosts. At the time we carried out the host-association study we selected for the presence or absence of the four marker genes (γ-glutamyl transpeptidase, *dmsA* (*cju34*), (*cj1585c*) oxidoreductases and a serine protease) in *C. jejuni* strains NCTC 11168 (Parkhill *et al*., 2000) and 81-176 (Hofreuter *at al*., 2006). We endeavoured to find differences in the distribution of these genes in *C. jejuni* populations as indicated by the different strains that were hosted by humans, chickens and bovines. Interestingly, we found that *ggt* and *dmsA* were found more often among chicken and humans strains than among those that infect bovines. In contrast, oxidoreductase (*cj1585c*) was found more often among bovine isolates than among those of chicken and humans. Serine protease was relatively common among bovine than among humans or chicken isolates. At the time the study was performed we did not have MLST data of the isolates. Some studies (Zautner *et al*., 2011) and the results of our present study (unpublished) show that these characteristics are strictly associated with certain STs. For example, clonal complex ST-45 is usually *ggt* positive and is common in human infections and chickens. In contrast, clonal complex ST-61, is common in bovines but which is uncommon in other hosts is also *ggt* negative (de Haan *et al*., 2010; Zautner *et al*. 2011). Several new studies using MLST data that is used for analyzes and mathematical modeling, e.g with STRUCTURE (Pritchard *et al*., 2000) or BABS (de Haan *et al*., 2010) have confirmed that certain ST types are host-associated. Thus, *C. jejuni* strains acquired certain characteristics when they colonize their host animals. Consequently, this host-bacterium adaptation has left these adaption signatures in their genomes, detected as differences in their genomes such as *ggt*, *dmsA* etc.

The γ-glutamyl transpeptidase (*ggt*) probably has an important role in *C. jejuni* colonization of the gut. Barnes *et al*., (2007), found that *ggt* was important in persistent colonization of chicken gut and that it catalyzes the conversion of gluthatione and glutamine to glutamate. They also found that *ggt* was not present in

all human and chicken *C. jejuni* strains, which agrees with what we found in our present study. The ability of some *C. jejuni* strains to use glutamine or gluthione as a sole carbon sources depends on the presence of *ggt* (Guccione *et al*., 2008; Hofreuter *et al*., 2008). The *ggt* gene has a pivotal role in combating oxidative stress by maintaining cellular gluthathione levels (Tate & Meister, 1981). We found only a low frequency of *ggt*-positive isolates 8.4% among bovine isolates, which suggests that this type of metabolism is not crucial for *C. jejuni* colonization of the bovine gut. Similarly only approximately 30% of chicken and human strains were *ggt* positive, which also indicates that this enzyme is not necessary for the successful colonization of these animals by *C. jejuni*.

When oxygen levels are low, *C. jejuni* has the capacity to utilise a wide range of electron acceptors, including fumarate, nitrate, nitrite, sulfite, trimethylamine-*N*-oxide (TMAO) and dimethyl sulfoxide (DMSO) (Sellars *et al*., 2002; Pittman & Kelly, 2005). The subunit *dmsA* (*Cju34*) is part of the putative tripartite anaerobic DMSO oxidoreductase gene. Moreover *Cj*1585*c* is a gene that encodes a putative oxidoreductase. The role of oxidoreductase enzymes is to catalyze the oxidation of one compound with the reduction of another (Weidner *et al*., 1993). This group of enzymes usually uses NADP or NAD as cofactors. In general *C. jejuni* present a branched complex electron transport chain capable of utilizing multiple electron donors and acceptors (Weingarter *et al*., 2008) and our results suggest flexibility in the oxidoreductase system among different *C. jejuni* strains as well.

The *cjj81176-1367/1371* entity denotes a gene that encodes a serine protease. In *C. jejuni* proteases have a role in stress tolerance, but it is not known yet whether serine protease is important in the pathogenesis of campylobacteriosis (Cohn *et al*., 2007). In our work, the serine protease gene was common among the bovine isolates.

Our later studies used the MLST methods for the same strains that were studied for marker genes and found to have host associations between certain STs. In a study by Kärenlampi *et al*. (2003), it was observed that the degree of overlap between human and chicken isolates was 61% whereas between human and bovine isolates it was only 5.7%. This finding agrees with our results, which showed that the frequencies of the four gene markers were more similar between chicken and human than between

bovine and human. In a study by de Haan *et al*. (2010), in which MLST has used ST type had associations with some hosts which had certain genetic lineages.

6.2 Reduction of *C. jejuni* counts on chicken meat treated with different seasoning combinations (III)

The reduction in *C. jejuni* counts in chicken meat that had been treated with different seasoning combinations was as much as 1.66-log of storage at 4°C after one week for seasoning combination F and 1.52-log for seasoning combination D. Both combinations contained sodium lactate and a fraction of potato protein which were not included in other seasoning combinations (A, B, C, and E). The reduction of *C. jejuni* counts was higher when the seasoning that contained a low- or high- molecular weight fraction of potato protein in combination with sodium lactate (D and F) was included. Sodium lactate (E325) is a food additive that is used frequently in seasoning meat and poultry products and it is recommended as a flavor enhancer in fresh cooked meat and poultry products (Igoe, 2011). The sodium lactate solution, which has a pH of 6.8 to 7.0, is used as a pH control agent, and additions of between 2 to 4% do not alter the meat pH (Alvarado & McKee, 2007). Lactate has also been reported to be effective as a firming agent and a humectant (Chen & Shelef, 1992) and has been report to have bactericidal properties (Bacus & Bontenbal, 1991). Rajkovic *et al*. (2010) obtained a reduction of 1.2 logCFU/g *C. jejuni* counts in chicken meat after it had been stored at 4°C for seven days inoculated with lactic acid buffered with sodium lactate. In our study, sample taken 1 h after being treated with sodium lactate (F), the log CFU/g decrease was more rapid than in the samples without the sodium lactate in the seasoning (E), which was a very rapid decrease.

Potato proteins are extracted from potato juice, and two different fractions are separated according to their molecular weights. The high molecular weight fraction contains a protein, patatin, as a major component, and the low fraction contains protease inhibitors (Pots *et al*., 1999; Rymareva *et al*., 2003). In our investigation, both fractions contributed to the reduction in *C. jejuni* counts. The bactericidal activity of the low-molecular weight fractions of potato proteins could be associated with the activities of protease inhibitors have on some bacterial proteins that are

important for the protection of *C. jejuni* cells against external stress factors (González & Hänninen, 2011). Molecules of antimicrobial polypeptides interact with the bacterial membrane which gives rise to the formation of a transmembrane cluster (probably, an ion channel). This causes a decrease in the membrane potential value and subsequent cytolysis (Molina *et al*., 1993). Other components in our seasoning combinations were thickening agents that absorbed water from the meat surface, which changed the seasoning from a dry powder to a gel covering the meat surface (potato starch (E1424), methylcellulose (E461), konjac gum (E425), xantham gum (E415)). These components had no discernable effects on the *C. jejuni* counts.

6.3 Death rate of *C .jejuni* on chicken meat treated with different seasoning combinations (III)

The mechanism for mediating a faster death rate observed in the beginning of the experiments on the seasoning combinations could be explained by a sequence by which those bacteria that were injured or stressed would die first. Another explanation for the initial maximum antibacterial effect shortly after treatment could be due to an inactivation of the bactericidal compounds of the seasoning after prolonged contact with the meat. Some studies that dealt with the survival of *C. jejuni* on frozen chicken meat stored at -20°C showed similar results to our findings with seasoning combinations. In those studies around decrease of 1 log in CFU/g was obtained soon after freezing and no significant reduction in counts were obtained thereafter (Bhaduri & Cottrell, 2004; Ritz *et al*., 2007; González *et al*., 2009; Sampers *et al*., 2010). In our **study III** the initial pH was lower in treated products (0.6-0.8) than in the untreated products, but it should be noted that similar after one day of storage. The pH is not the only factor to explain the 0.8 to 1.4 log CFU/g differences in the death rate of *C. jejuni* between untreated and treated with seasoning products.

Recent risk assessment performed by EFSA with *C. jejuni* in chicken meat and the risk of campylobacteriosis has concluded that a two-log reduction of CFU/g in chicken meat reduced human risk of campylobacteriosis by 50 to 90% (EFSA BIOHAZ 2011).

6.4 Modelling the survival of *C. jejuni* in minced chicken meat and well water at different temperatures (II, IV)

Our data show that the Weibull model is a useful tool in the modeling of *C. jejuni* survival both in minced chicken meat and in well water over a wide range of temperatures. These food matrices have highly variable survival characteristics for *C. jejuni*. The reason for preferring the Weibull model over the traditional log-linear model is because the Weibull model is more reliable over wider ranges of temperatures. An accurate fit is essential to obtain reliable predictions, which render the Weibull model more suitable for practical use than the log-linear model (Albert & Mafart, 2005).

Our data (**study II)** revealed that *C. jejuni* survived longer at -20°C than at any other temperature tested in minced chicken meat for up to 56 days. The *C. jejuni* counts reached the detection limit of 1 log CFU/g. Garénaux et al. (2009) proposed that some strains were capable of developing a mechanism to resist potential stress factors associated with the bacterial cell inactivation during cold storage of foods. The results of (**study IV**) also showed longer survival in water at refrigeration temperatures, at around 4°C. This finding is supported by previous data on survival of *C. jejuni* in water (John & Rose, 2005; Tatchou-Nyamsi-Konig *et al*., 2007). Cools *et al*. (2003) indicated that maximum time for the detection of *C. jejuni* in water to be at 4°C could vary from 2 to 4 weeks to more than 4 months. These differences were attributed to different strains, differences in experimental conditions or in the methodology of culturability assessment. Guillou *et al*. (2008) also showed that the loss of *C. jejuni* culturability observed in all conditions tested was shown to be dependent on the strain, preculture conditions and the water composition.

6.5 Effects of antibiotic resistance on survival of *C. jejuni* (II, IV)

The antimicrobial resistant variants 49/7RAT and 49/7RATCIP32 had significantly ($P < 0.05$) longer survival than did the wild-type strain 49/7R over the whole range of temperatures (-20°C to 25°C) in minced chicken meat (**study II**). The results of (**study IV)** revealed that the antimicrobial resistance, mainly to ciprofloxacin that

ocurred, may also affect the survival of *C. jejuni* strains in well water. Ciprofloxacin resistance increased the survival of *C. jejuni* strain ATCC 33560CIP32 but decreased the survival of the variant 49/7RAT and 49/7RATCIP32. Luo *et al*., 2005 indicated that fluoroquinolone resistance (ciprofloxacin is a fluoroquinolone) improved the fitness of a *C. jejuni* strain in chicken intestinal colonization. Recently Zeitouni & Kempf (2011) found that ciprofloxacin resistance-associated fitness seems to be a strain dependent characteristic. In addition, Petersen *et al*. (2009) showed that fitness cost caused by antimicrobial resistance in *Escherichia coli* varied with environmental conditions it was tested. These results fit with our results on *C. jejuni* that effect of antimicrobial resistance on survival is both strain and matrix dependent.

7. CONCLUSIONS

1. Our results revealed that the genes associated with amino acid metabolism (*ggt*) and electron transfer (*cj*1585*c* an*d dmsA*), colonization (*ggt*), or unknown function (serine protease gene) were not randomly distributed among the isolates from different hosts and enabled the assignment of the chicken or bovine as sources of *C. jejuni* isolates. Although, in nature colonization by *C. jejuni* is multifactorial, the identification of bacterial colonization factors may enable the development of strategies for intervention, prevention or control of campylobacteriosis in the future. These results suggest that metabolic diversity is an important adaptive factor in host adaptation.

2. The ability to predict the survival characteristics of *C. jejuni* will improve food safety and provide a useful tool for the development of a better risk assessment in controlling *C. jejuni* in chicken meat and well water. The Weibull model is a useful tool in the modeling of *C. jejuni* survival in chicken minced meat for a wide temperature range from (-20°C to 25°C) and in well water (from 4°C to 25°C).

3. Antimicrobial resistance increased the survival of a *C. jejuni* strain on minced chicken meat. The antimicrobial resistance pattern of *C. jejuni* influenced *C. jejuni* survival in well water, either by impairing the fitness or by enhancing fitness, and consequently survival. More research is needed to understand better the effect of strain variation on the survival mechanisms of *C. jejuni* in foods and drinking water.

4. When chicken legs were treated with different seasoning combinations the greatest decrease in *C. jejuni* CFUs were obtained during the first hours after the treatment. This finding indicated that the risk of *C. jejuni* exposure for consumers of treated meat chicken is reduced early on after treatment. This is important for consumer risk because risk reduction is achieved at the same time as when products are at retailed. The sell-out date of marinated chicken meat products in Finland is 10 days. The decrease of *C. jejuni* counts of a maximum 1.66 log CFU/g by seasoning combinations used in our studies would reduce the risk for human infection considerably.

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